GROWTH AND SURVIVAL OF ENTEROBACTERIA IN SELENITE-CYSTINE BROTH CONTAINING THIOSULFATE

SUMMARY—Concentrations of 0.1 or 0.2% selenite-cystine and 0.16 or 0.32% thiosulfate in trypticase soy broth were bactericidal for seven strains of Escherichia coli, *a* Shigella *sp. culture, and* Salmonella cholerae-suis. *Higher concentrations of both reagents resulted in the inhibition of six strains of* Salmonella *and a culture of* Arizona *sp. The growth and length of the lag period of cultures of* Enterobacter, Klebsiella, Proteus *and* Pseudomonas *were not significantly affected. In mixed cultures of* E. coli *and* Salmonella enteritidis, E. coli *was selectively inhibited by the selenite-cystine-thiosulfate mixture even when present in large numbers. Uptake of selenium-75 by intact and spheroplast cells of* E. coli *and* S. enteritidis *in selenite-cystine medium in the presence and absence of thiosulfate was not significantly different. It was concluded that the cell wall does not play a major role in the uptake of selenite by these organisms. The formation of "seleno-polythionates" has been demonstrated in the selenite-cystine-thiosulfate mixture. Use of this medium to isolate* Salmonella *from contaminated food samples indicated that it was not as effective as commercial selenite-cystine broth or tetrathionate broth.*

INTRODUCTION

THE ADDITION of sodium selenite to microbial growth medium was first reported by Leifson (1936) and has since been used widely for the routine isolation of salmonellae. The mechanism responsible for the selectivity of this medium is not understood, although selenium has been reported (Cowie and Cohen, 1957; Scala, 1964; Skarzynski and Szczepkowski, 1959) to impair protein synthesis by *Escherichia coli*. Evidence also exists for a nonenzymatic reaction by which microorganisms may be inhibited by selenium compounds (Scala and Williams, 1962; Weiss et al., 1965).

The inhibition of enterobacteria by tetrathionate broth also is well known and utilized in selective media. It has been demonstrated recently that both thiosulfate and tetrathionate are necessary to obtain the inhibitory effect, while other polythionates and inorganic compounds present in tetrathionate broth exert no significant effect upon the growth of enterobacteria (Palumbo and Alford, 1970). Because sodium selenite has been theorized to react with certain inorganic sulfur compounds to form seleno-polythionates (Smith, 1959), the present study was designed to determine if seleno-polythionates are formed in a selenite-cystine-thiosulfate mixture, to examine the effect of the mixture on the growth of enterobacteria and on the uptake of selenium, and to examine the usefulness of selenite-cystine-thiosulfate broth mixture as a possible selective medium.

MATERIALS & METHODS

Bacterial strains, media, and chemicals

The bacterial strains used in this study were obtained from our stock collection. The strains were routinely cultured on Baltimore Biological Laboratories' (BBL) trypticase-soy broth (TSB). Tetrathionate broth, selenite-cystine broth, Brilliant Green agar, Triple Sugar Iron agar, Purple Lactose broth, and the serological reagents were all obtained from Difco. L-Cystine (0.025g) (Nutritional Biochemicals) was dissolved in 1 ml of 1N NaOH. To this solution were added 70 ml distilled water and 10g sodium selenite (BBL). The pH was adjusted to 7.0 with 1N HCl, the final volume adjusted to 100 ml with distilled water, and the solution sterilized by filtration through a membrane filter (Millipore Corp., 0.45μ pore size). We prepared thiosulfate as a 32.8% solution in distilled water and sterilized it by autoclaving. Bacteria also were cultured in minimal salts solution (MSS), prepared as described by Cowie et al. (1951) except that $MgSO_4$ was replaced by $MgCl_2$ to produce a low sulfur medium. When 20% sucrose medium was needed for experiments employing spheroplast cells, equal amounts of 2× TSB or MSS were mixed with a 40% sucrose solution, which was sterilized by autoclaving separately to minimize caramelization. Sodium selenite-75 was obtained from Amersham-Searle, Des Plaines, Ill., and added directly to the medium immediately before inoculation.

Bacterial growth measurements

Working stock cultures of bacteria were prepared by inoculating tubes containing 9 ml of TSB from stock slants and incubating at 37°C overnight. After incubation, 0.1 ml of the culture was added to a tube containing 9 ml of TSB and appropriate amounts of selenite-cystine and thiosulfate. The cultures were incubated at 37°C and assayed at intervals by standard plate counts. All plating was done in triplicate. Resting cell suspensions were prepared by centrifuging 50 ml of an overnight culture of each organism at 10,000 rpm for 5 min. The cell pellets were washed once with 50 ml of distilled water, recentrifuged, and resuspended in 100 ml 0.125M phosphate buffer which contained 0.4% selenite-cystine and 0.2% thiosulfate. The suspensions were incubated at 37°C and assayed at intervals by standard plate counts.

Mixed cultures were assayed by the three-tube, three-dilution, most-probable-number method (Galton et al., 1968), using Purple Lactose broth. Because the lactose-negative salmonellae grew to a much higher final concentration in the selenite-cystine-thiosulfate medium than did the lactose-positive *E. coli*, ready identification was possible in Purple Lactose broth.

Preparation of spheroplast cells

Overnight broth cultures of the stock strains were used to inoculate flasks containing 100 ml of TSB. The flasks were incubated at 37°C overnight and the cultures centrifuged at 10,000 rpm for 3 min; the bacterial pellets were resuspended in 100 ml of TSB or MSS containing 20% sucrose and 2,000 units of penicillin G per ml, and incubated for 4 hr at 30°C in a waterbath-shaker at 75 rpm. Spheroplast formation was confirmed by microscopic examination of a smear or wet mount from such cultures and by loss of optical density upon 10-fold dilution in water.

Radioactivity measurements

Bacterial uptake of sodium selenite-75 was measured in a liquid scintillation spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill., Model 6725). Bray's scintillation medium, containing 30g naphthalene, 10 ml ethylene glycol, 50 ml methanol, 2g 2,4-diphenyloxazol, and 0.1g 1,4-bis[2-(5-phenyloxazolyl)]-benzene to a final volume of 500 ml with dioxane, was used. Quenching was monitored using an internal standard which indicated that no corrections were necessary.

When cultures of intact or spheroplast cells were incubated in the presence of selenite, metallic selenium was often precipitated outside the cells. To distinguish precipitated selenium from that taken into the cells, cultures were layered over 40% sucrose and centrifuged at 10,000 rpm for 3 min (for intact cells) or 5,000 rpm for 10 min (for spheroplast cells). Following centrifugation, the sucrose layer, which contained bacterial cells or spheroplasts in suspension, was assayed for protein and radioactivity. The upper layer of medium and the pellet of metallic selenium were discarded. Because the addition of thiosulfate to the medium resulted in a marked decrease in cells in some cultures, viable cell counts were useless for comparison with radioactivity uptake measurements. Therefore, the uptake of selenium in each culture was expressed as a function of the protein content of the sucrose layer following centrifugation. The spheroplast cells were assayed directly for protein by the method of Lowry et al. (1951) while the protein content of intact cells was derived on assay of material obtained after disruption of intact cells by sonification (Branson Sonifier, Model S-125).

Chromatographic analysis

To determine if polythionates were present in the selenite-cystine-thiosulfate mixture, 9 ml of TSB containing 0.8% selenite-cystine, 1.28% thiosulfate, and trace amounts of sodium selenite-75 were incubated at 37°C overnight, filtered (Millipore Corp., 0.45μ pore size) to remove precipitated selenium, and 0.01 ml of the filtrate was spotted onto a sheet of Whatman 3MM chromatographic paper. Chromatographic separation was performed according to the method of Skarzynski and Szczepowski (1959), in a closed vessel in a solvent of n-butanol-acetone-water in a 2:2:1 ratio. In approximately 2 hr the solvent front had advanced 8 in. and a good clear separation was obtained. The chromatograms were air dried, stained in 1.7% (w/v) silver nitrate, washed successively with tap water, 5% ammonium hydroxide, and with tap water, and air dried. The spots were cut from the chromatogram, cut into small pieces, mixed with 20 ml of Bray's solution, and assayed for radioactivity.

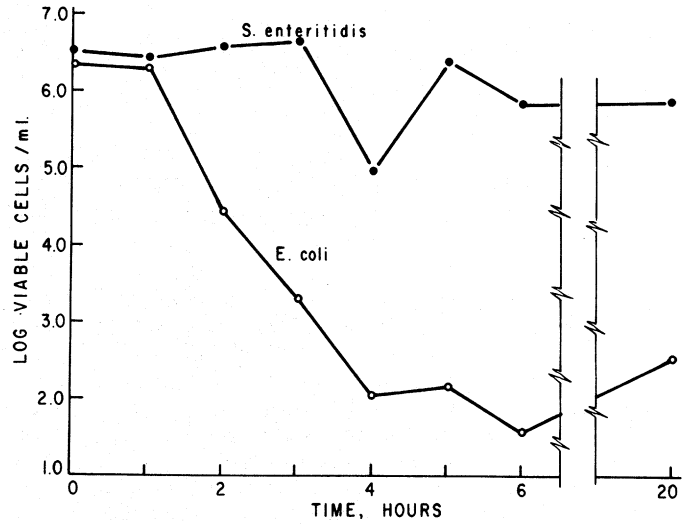

Fig. 1—Effect of 0.6% thiosulfate upon survival of Escherichia coli and Salmonella enteritidis in selenite-cystine broth at 37° C.

RESULTS

Effect of selenite-cystine-thiosulfate on survival of E. coli and S. enteritidis

E. coli and *S. enteritidis* were cultivated at 37°C in selenite-cystine broth containing thiosulfate in a final concentration of 0.64% so that equimolar amounts of $NaHSeO_3$ and $Na_2S_2O_3 \cdot 5\text{-}H_2O$ were obtained. Under these conditions, as shown in Figure 1, *E. coli* cultures decreased almost 5 log cycles within the first 6 hr, while *S. enteritidis* showed a drop of only one-half of a log cycle. Thiosulfate added to TSB had no effect upon the survival of either culture in the absence of selenite-cystine. Cultivation in selenite-cystine broth alone at 37°C overnight produced no significant inhibition of *S. enteritidis* and caused a drop of approximately one-half of a log cycle in the overnight growth of *E. coli*.

In a similar experiment using a resting cell suspension, the inhibitory effect of the selenite-cystine-thiosulfate mixture upon *E. coli* was insignificant during the first 6 hr and resulted in a total decrease of only 2 log cycles after 24 hr incubation at 37°C.

Effect of various concentrations of selenite-cystine-thiosulfate on growth of E. coli and S. enteritidis

It can be seen in Figures 2 and 3 that TSB containing selenite-cystine and thiosulfate in final concentrations at or below 0.2% and 0.32%, respectively, exerted no inhibitory effect on the growth of *S.*

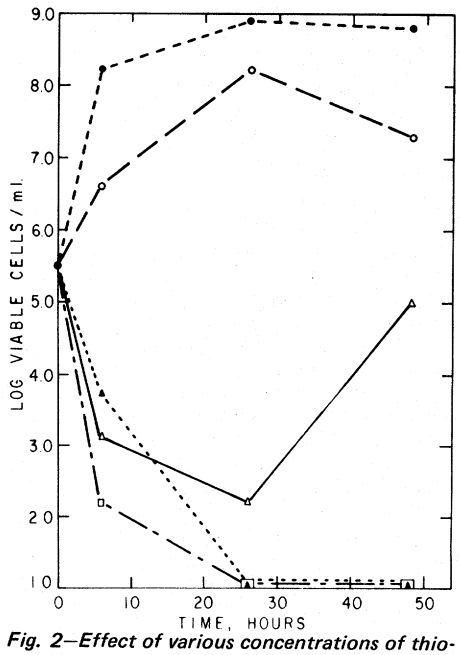

Fig. 2—Effect of various concentrations of thiosulfate and selenite-cystine upon growth and survival of Escherichia coli in trypticase-soy broth at 37° C. (Legend: ○ Control (no additions); ● 0.1% SC; 0.16% T; △ 0.2% SC; 0.32% T; ▲ 0.4% SC; 0.64% T; and □ 0.8% SC; 1.28% T

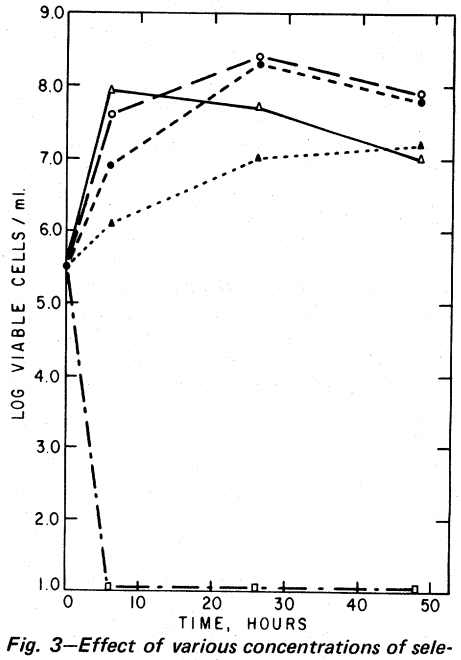

Fig. 3—Effect of various concentrations of selenite-cystine and thiosulfate on growth and survival of Salmonella enteritidis at 37° C. (Legend: see Fig. 2.)

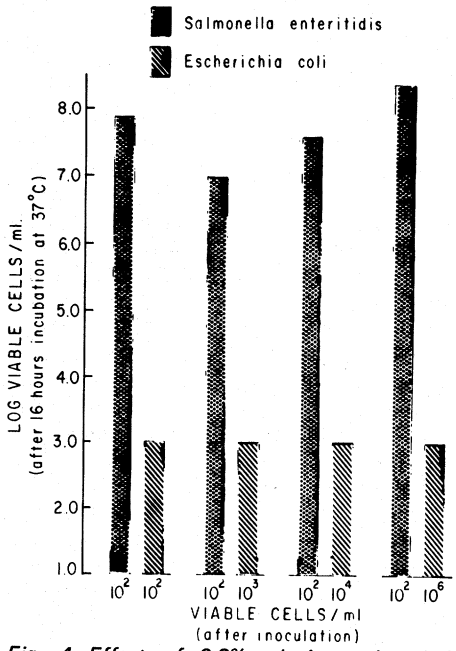

Fig. 4—Effect of 0.2% selenite-cystine and 0.32% thiosulfate on survival of Escherichia coli and Salmonella enteritidis in mixed culture.

enteritidis but did significantly decrease the growth of *E. coli* when compared to control cultures grown in TSB alone, while concentrations of selenite-cystine and thiosulfate equal to or greater than 0.4% and 0.64%, respectively, significantly inhibited the growth of both cultures.

Effect of selenite-cystine-thiosulfate on growth of mixed cultures of E. coli and S. enteritidis

E. coli was mixed with *S. enteritidis* in ratios 1:1, 10:1, 100:1, and 10^4:1 and inoculated into TSB containing 0.2% selenite-cystine and 0.32% thiosulfate. In every case, the effect was selectively bactericidal for *E. coli* while allowing the growth of *S. enteritidis* (Fig. 4).

Effect of selenite-cystine-thiosulfate on growth and survival of various enterobacteria

The effect of TSB containing 0.2% selenite-cystine and 0.32% thiosulfate on representative cultures of enterobacteria is summarized in Table 1. The data show that the medium was significantly bactericidal for all of the *E. coli* cultures tested. With one exception, the *Salmonella* cultures tested increased beyond the level at which they were inoculated (approximately 10^6 organisms/ml). *S. cholerae-suis*, which closedly resembled the *E. coli* cultures in its biochemical characteristics, was also significantly inhibited by these concentrations of selenite-cystine-thiosulfate. Of the other genera tested, only the *Shigella* culture was significantly inhibited, while *Enterobacter, Pseudomonas, Klebsiella* and *Proteus* cultures showed a significant increase in cell numbers. When concentrations of selenite-cystine and thiosulfate were doubled to 0.4% and 0.64%, there was no inhibitory effect upon *Pseudomonas* and *Proteus* cultures. The inhibitory effect upon *Enterobacter* and *Klebsiella* cultures at these higher concentrations was variable but not as great as the inhibition of *Salmonella* at these concentrations.

Although several of the cultures tested in the experiments described above were not inhibited by 0.2% selenite-cystine and 0.32% thiosulfate with respect to total growth, the effect of these reagents upon the length of the lag period was examined. In such experiments, growth of *Enterobacter aerogenes* in broth containing 0.2% selenite-cystine and 0.32% thiosulfate was compared to growth of *S. enteritidis* in the same medium by performing viable cell counts at frequent intervals. The lag period of *E. aerogenes* was not affected on culturing in selenite-cystine-thiosulfate broth when compared to that of *S. enteritidis* in the same medium. There also was no difference in the length of lag periods of these organisms when grown in selenite-cystine broth alone. Similarly, there was no effect upon the lag periods of *Proteus, Pseudomonas*, or *Klebsiella* cultures which were grown in selenite-cystine-thiosulfate broth when compared to either the lag periods of similar cultures in selenite-cystine broth alone or to the lag period of *S. enteritidis* in selenite-cystine-thiosulfate broth.

Chromatography of selenite-cystine-thiosulfate mixtures

Paper chromatographic separation of uninoculated selenite-cystine-thiosulfate mixtures, which had been incubated for 1 hr or 24 hr at 37°C, demonstrated the presence of thiosulfate, trithionate, tetrathionate, pentathionate, and, possibly, hexathionate. Radioisotopic selenite was incorporated in the mixtures in trace amounts. The spots were cut from the chromatogram and counted for radioactivity. Radioisotopic selenium was present in each of the polythionate spots (Table 2).

Effect of thiosulfate on uptake of selenium-75 by intact and sheroplast cells of E. coli and S. enteritidis

The uptake of $NaHSe^{75}O_3$ per micro-

Table 1—Effect of 0.2% selenite-cystine and 0.32% thiosulfate on growth and survival of various enterobacteria in trypticase-soy broth after incubation at 37° C for 16 hr

Organism	Avg viable cell count/ml[a]
Escherichia coli A	1×10^1
E. coli B	46×10^1
E. coli C	1×10^0
E. coli D	38×10^1
E. coli E	22×10^1
E. coli AA	7×10^0
Salmonella senftenberg	1.4×10^7
S. enteritidis	1.4×10^7
S. typhimurium	1.3×10^7
S. chester	2.2×10^7
S. derby	4.5×10^6
S. thompson	6×10^6
S. cholerae-suis	0 on 10^5
Enterobacter aerogenes	$>10^9$
Arizona sp.	$>10^9$
Pseudomonas sp.	$>10^9$
Klebsiella sp.	6.7×10^8
Proteus sp.	$>10^9$
Shigella sp.	0 on 10^3

[a]Inoculum approximately 10^6 viable cells/-ml.

Table 2—Paper chromatography of trypticase-soy broth containing 0.8% selenite-cystine, 1.28% thiosulfate, and trace amounts of sodium selenite-75

Thionate spot[a]	Selenium-75, counts/min
Thiosulfate	28,543
Trithionate	8,695
Tetrathionate	683
Pentathionate	306
Background	35

[a]As determined by comparison with a known polythionate mixture.

Table 3—Uptake of selenium-75 by intact and spheroplast cells of Escherichia coli and Salmonella enteritidis in trypticase-soy broth containing 0.32% thiosulfate and/or 0.2% selenite-cystine

Culture	Counts/min/gamma cell protein after 2 hr incubation in SC	SCT
E. coli intact cells	29	10
E. coli spheroplast cells	30	19
S. enteritidis intact cells	20	17
S. enteritidis spheroplast cells	30	22

Table 4—Isolation of salmonellae from bone meal[a] in trypticase-soy broth containing 0.32% thiosulfate and/or 0.2% selenite-cystine

0-serogroup	No. positive after 24-hr growth in SC	SCT	No. positive after 48-hr growth in SC	SCT
A	1	2	1	1
B	1	1	2	2
C_1	22	9	35	7
E	1	3	2	3
Unidentified	16	9	19	11
Total	41 (41%)	24 (24%)	59 (59%)	24 (24%)

[a]100 1-g samples were inoculated into each type of medium.

gram of cell protein by organisms cultured in selenite-cystine broth (final concentration of selenite 0.2%) was compared to the uptake in selenite-cystine-thiosulfate broth (final concentrations, 0.2% selenite-cystine and 0.32% thiosulfate) for both intact and spheroplast cells. The results of a typical experiment are presented in Table 3. Statistical analysis of four such experiments indicated that the uptake of selenium for intact and spheroplast cells was not significantly different ($P > 0.5$) in the presence or absence of thiosulfate.

Comparison of media for isolation of salmonellae from contaminated food samples

Since the data with mixed cultures of *Escherichia* and *Salmonella* indicated that selenite-cystine-thiosulfate medium was selective for the isolation of salmonellae, a series of naturally contaminated samples was examined. The percentage of serologically-positive *Salmonella* isolations from a contaminated bone meal sample, using commercial selenite-cystine broth, was compared to the percentage using broth containing 0.2% selenite-cystine and 0.32% thiosulfate. 100 1-g samples were inoculated into 10-ml broth tubes of each medium. Results of this experiment, summarized in Table 4, demonstrated that commercial selenite-cystine medium was more effective than the selenite-cystine-thiosulfate mixture.

Similarly, the isolation of *Salmonella* in selenite-cystine-thiosulfate broth and in tetrathionate broth was compared using contaminated samples of dried egg albumen. Following the BAM procedure described by Elliott (1966), 1-g samples were pre-enriched in lactose broth for 24 hr at 37°C before inoculation into selenite-cystine-thiosulfate broth and tetrathionate broth. After 24 hr and 48 hr in these media, samples were examined for salmonellae by streaking on Brilliant Green agar, picking colonies to Triple Sugar Iron agar and confirming serologically with Salmonella 0 antiserum (poly A to I), 0 group antisera (A to E), and H antiserum (poly a to z). Although an insufficient number of samples was examined to permit a statistical evaluation, the data indicated that, in each case, use of tetrathionate broth was more effective than the selenite-cystine-thiosulfate mixture.

while the addition of thiosulfate to selenite-cystine broth results in a marked bactericidal effect on *E. coli*, it did not render the medium more effective than either selenite-cystine or tetrathionate broth in selecting *Salmonella* organisms from contaminated samples. It is possible that *Salmonella* organisms contaminating the samples tested were damaged by commercial treatment and prolonged cold storage; however, even pre-enrichment of samples in lactose broth according to the BAM procedure did not result in an increase in the efficiency of isolation of *Salmonella* cultures by selenite-cystine-thiosulfate broth. The ineffectiveness of the selenite-cystine-thiosulfate medium was not caused by the specific inhibition of a single *Salmonella* serotype contaminating the samples, since there was a wide variety of serotypes present in the samples. Thus, from a practical standpoint, the use of selenite-cystine medium is still recommended for isolation of *Salmonella* sp. However, the failure of even relatively high concentrations of selenite-cystine-thiosulfate to inhibit cultures of *Proteus* and *Pseudomonas* would suggest that the medium might be useful for the selective isolation of these organisms.

Pardee and Watanabe (1968) have demonstrated that the sulfate binding protein of *Salmonella typhimurium* is located in the wall-membrane region. They support their conclusions by the lack of the binding protein in spheroplast or osmotically-shocked cells, and also by the ability of the binding protein to bind sulfate in bacteria which cannot transport sulfate into the cell. Since sulfur and selenium metabolism often seem to be related, the uptake of selenium in selenite-cystine and selenite-cystine-thiosulfate broth by both intact and spheroplast cells of *E. coli* and *S. enteritidis* was examined, but was not significantly different. It would appear that the cell wall does not play a major role in selenium uptake. Although the metabolic effects of selenium are well known, it is possible that the entrance of selenium into the cell is a nonenzymatic reaction. If the entrance of selenium into the cell is nonenzymatic, one would expect the survival of resting cells to be similar to that of actively metabolizing cells. However, the finding that the inhibitory effect of the selenite-cystine-thiosulfate broth was much greater on actively metabolizing cells than on resting cells lends support to the view that the effect of selenium is primarily metabolic. Chromatographic analysis of selenite-cystine-thiosulfate broth has indicated the presence of "seleno-polythionates." These data agree well with the findings of Smith (1959) for the production of seleno-polythionates from selenite and thionates. Selenite is known to react with sulfhydryl groups and, recently, Parker and Allison (1969) demonstrated that tetrathionate can inactivate glyceraldehyde 3-phosphate dehydrogenase by combining with the catalytically active sulfhydryl group of the enzyme. Perhaps the formation of seleno-polythionates, while not increasing the nonenzymatic uptake of selenium and its compounds, makes them more effective once within the cell so that they are able to enter into the metabolism of the cell on a more competitive basis.

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Trade names are mentioned for identification, implying no endorsement.